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In vitro conservation of apical meristem-tip of *Melia azedarach* L. (*Meliaceae*) under slow-growth conditions

(with 1 table & 2 figures)

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Abstract. Studies on *in vitro* storage of *Melia azedarach* –"paradise tree"- under slow-growth conditions were carried out to develop an efficient protocol for the conservation of the genetic diversity of the crop. The response to different growth retardation treatments was examined using clone E. *In vitro* cultures of apical meristem-tips (0.2-0.3 mm in length) could be effectively maintained for 12 months at 4ºC on ¼MS basal medium supplemented with 0.5μM BA. Shoot proliferation was observed on 67% of the recovered apical meristem-tips, after 12 months at 4ºC. Rooting of the regenerated shoots was achieved by reculturing them on MS + 17.5 μM IBA, during 4 days and subsequently transferred to MS medium lacking growth-regulators for 30 days. Complete plants were successfully transferred to the greenhouse.

Keywords: conservation, germplasm, in vitro culture, Melia azedarach, meristem

Among the *in vitro* techniques for the conservation of plant germplasm, slow growth has been used as a medium-term storage option with subculture intervals to be extended between 12 months and 4 years. Large collections are currently stored in slow growth such as, cassava, potato, *Musa* spp., yam (5).

Physical conditions of incubation and/or modifications of the culture media which reduce the growth rate of the tissues stored *in vitro* are

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usually employed for the preservation of plant germplasm. Although the most successful and widely procedures are based on either, reduction of temperature of incubation or reduction of the concentrations of the chemical components of the media culture (10), procedures which combine reductions of temperature and incubation in the darkness have been also useful (2, 8, 9).

The “paradise tree” (*Melia azedarach* L. family *Meliaceae*) is indigenous to Southern Asia. It has been introduced to subtropical areas of Argentina where it is one of the most valuable timber trees. The attraction of this species desides the quality of its timber as well as its fast rate of growth. “Paradise tree” is conventionally reproduced through seeds, which results in highly heterozygous populations. Therefore, asexual techniques of propagation were developed for multiplication of selected clones, including micropropagation using nodal explants (1, 4, 11) and meristem cultures (12). However, very little work has been done to preserve *in vitro* germplasm of the selected clones. It has been reported (3) that after 4 months of storage at 4°C (using 200μM salicylic acid), as much as 83% of the embryonic axes were successfully recovered. Although seed conservation at –20°C was also tried, only 43% of the seeds germinated at the end of the experiment (6).

This investigation was designed to study the survival of one clone of *Melia azedarach* L. after storage of meristems, cultured in several media, at 4ºC for 4, 8 and 12 months without subculture.

MATERIALS & METHODS

Plant material. The clone E of “paradise tree” (*Melia azedarach* L.) obtained from Danzer Forestación S.A. (Posadas, Misiones, Argentina) was used in this study. *In vitro* establishment of a stock of source of explants was initiated from axillary buds from an adult tree (more than 10 years old) as mentioned by (4), consisting of: a) disinfection of explants with 70% ethanol (3 min), followed by immersion in NaOCl (2% active Cl) with 0.1% Triton-X-100® (20 min) and finally rinsed three times with sterile distilled water. b) Establishment of culture on MS basal medium (7) supplemented with 2 μM BA (6-benzylaminopurine), 0.5μM IBA (indolebutyric acid), 0.3 μM GA_3 (gibberellic acid) and c) Multiplication of the regenerated shoots in MS + 2μM BA + 0.3μM GA_3. All media were solidified by using 0.7% Sigma agar (A-1296). The pH of the media was adjusted to 5.7 with KOH or HCl prior to adding the agar. The media were dispensed in glass tubes 40 cc or glass jars of 80 cc, obturated with aluminium foil and autoclaved at 1.46 $Kg.cm^{-2}$ for 20 min.

The cultures were incubated in a growth room at 27 ± 2°C (14 hs. photoperiod with an irradiance of 4.5$\mu mol.m^2.s^{-1}$ provided by cool white fluorescent lamps).

Protocols for culture and conservation of apical meristem-tips. The apical meristem-tips (0.5-0.7 mm in length, consisting of the domo and a pair of leaf primordia) were cultured in 11 cc glass tubes containing 3 cc of nutrient medium. The media were composed either of MS, or half (1/2MS), or quarter strength (1/4MS). These media were supplemented with different combinations and concentrations of IBA and BA (Table 1), comprising the medium (12) considered for meristem growth optimum (MS + 2µM BA + 0.5µM IBA) as well as other 20 suboptimum media with reductions in the basal medium salts and/or reduction of the concentrations of growth regulators (4 to 10 times less than the concentrations used in the optimum medium). All the media were prepared as described above. The tubes were sealed with Resinite AF-50® (Casco S.A.I.C. Company, Buenos Aires). The cultures were incubated in a refrigerator in continuous darkness at 4°C.

Table 1.– Effect of 21 culture media used during three period of storage at 4ºC on shoot regeneration of *Melia azedarach* when subcultured on MS + 2µM BA + 0.5µM IBA at 27ºC during 60 days.

Treatment			% explant forming shoot after subculture in optimum medium		
			Months after storage		
	BA (µM)	IBA (µM)	4	8	12
MS	2	0.5	57(±5.7)^{ab}	40^{abcd}	0^{a}
MS	—	—-	87(±5.7)^{cd}	33(±1.6)^{ab}	0^{a}
MS	0.5	0.05	56(±10.9)^{ab}	43(±2.5)^{bcde}	0^{a}
MS	2	—-	42(±4.4)^{a}	37(±7.8)^{abc}	0^{a}
MS	—	0.5	100^{d}	36.7(±5.7)^{abc}	0^{a}
MS	0.5	—-	83(±11.5)^{cd}	43(±5.3)^{bcde}	0^{a}
MS	—	0.05	93(±3.7)^{cd}	47(±2.6)^{bcdef}	0^{a}
1/2MS	2	0.5	90(±10)^{cd}	43(±5.3)^{bcde}	0^{a}
1/2MS	—	—-	90(±10)^{cd}	47(±5.5)^{bcdef}	0^{a}
1/2MS	0.5	0.05	90^{cd}	50^{bcdef}	0^{a}
1/2MS	2	—-	90(±10)^{cd}	53(±5.4)^{bcdefg}	0^{a}
1/2MS	—	0.5	87(±5.7)^{cd}	60(±10)^{defg}	43(±4.3)^{c}
1/2MS	0.5	—-	90^{cd}	43(± 3.7)^{bcde}	0^{a}
1/2MS	—	0.05	100^{d}	56(±3.9)^{cdefg}	47(±4.7)^{cd}
1/4MS	2	0.5	74(±11.5)^{bc}	20(±10)^{a}	0^{a}
1/4MS	—	—-	93(±5.4)^{cd}	63(±2.7)^{efg}	43(±5.1)^{c}
1/4MS	0.5	0.05	92(± .6)^{cd}	66(±4.3)^{fg}	20(±10)^{b}
1/4MS	2	—-	83(±5.7)^{cd}	56(±1.6)^{cdefg}	47(±3.3)^{cd}
1/4MS	—	0.5	100^{d}	56(±4.9)^{cdefg}	61(±7.6)^{de}
1/4MS	0.5	—-	85(±5.7)^{cd}	73(± 4.2)^{g}	67(±3.6)^{e}
1/4MS	—	0.05	100^{d}	66(± 4.3)^{fg}	63(±3.3)^{de}

Similar letters, within the column, means no significant difference for Tukey's Multiple Comparison Test ($P < 0.01$).

Viability test of meristem maintained under slow-growth conditions. The cultures initiated at 4ºC were separated in three lots, which were recultured (at 27°C, 14 hs. photoperiod with an irradiance of 4.5µmol.m^2.s^{-1}), after 120, 240 and 360 days of storage respectively, on MS + 2µM BA + 0.5µM IBA (12) fresh medium with the optimum compositions to achieve shoot regeneration (Fig. 1). The cultures were done in a 80 cc glass-flask containing 25 cc of medium.

After 60 days of culture meristem viability was evaluated by recording the number of meristems capable of resuming growth and producing well developed shoots (with more than 25 mm long and with at least 6 expanded leaves).

Root induction. Root induction was obtained when the regenerated shoots were cultured on MS supplemented with 17.5µM IBA during four days and subsequent root formation in MS lacking growth regulators.

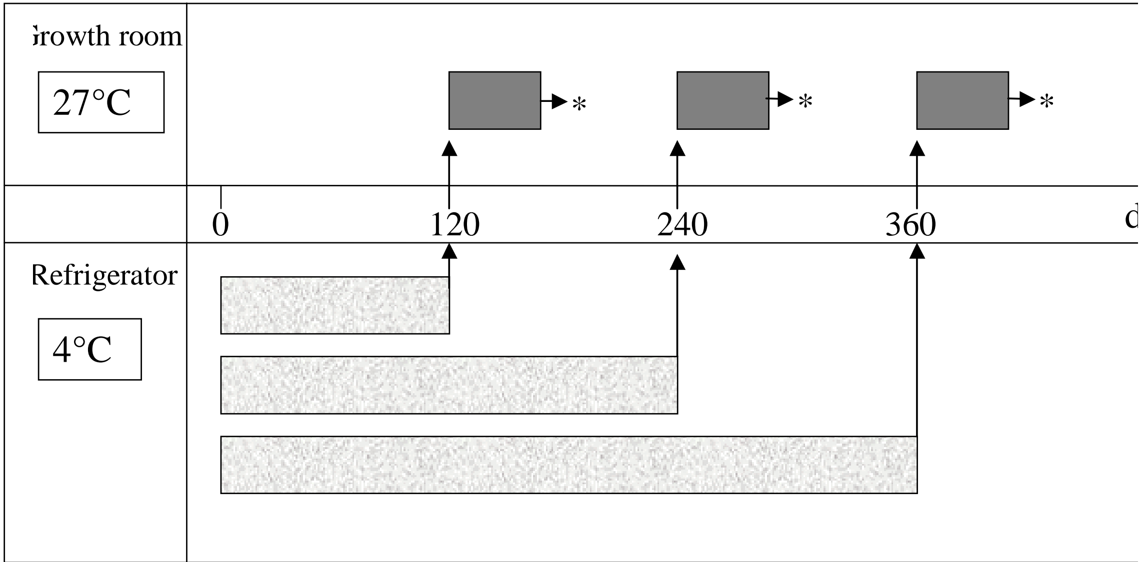
Experimental design & Statistical analysis. The experiment was performed with 10 apical meristem-tips for each treatment with three replications per treatment. Treatments were arranged randomly in the refrigerator and subsequently in the growth room.

The results presented in Table 1 are the mean of these replications with the standard error (± SEM). All data were subjected to analysis of variance (ANOVA) and comparisons of means were made with Tukey's Multiple Comparison Test ($P < 0.01$). The significance test was done by comparing the means among them.

RESULTS & DISCUSSION

Practically 100% of the meristems cultured as control on MS + 2 µM BA + 0.5 µM IBA, at 27°C, produced shoots with an average of 4-5 shoots/explant (data not shown). These results were almost identical to the ones informed previously for clone "E" (12).

The establishment of apical meristem-tips was successful. The contamination rates with microorganisms (bacteria and/or fungi) was very low (less than 2% of the cultures). The meristems remained green or pale green during storage. Practically they did not increase in length and never produced shoots at 4°C. However, when meristem stored for 120, 240 or 360 days were recultured on fresh medium (MS + 2µM BA + 0.5µM IBA) and incubated in light at 27°C, some of them remained alive and rapidly produced shoots. The percentage of meristems forming shoots depended on the duration of storage and of the composition of the medium. (Table 1). All the meristems cultured and stored during 120 days, independently of the media employed, produced shoots with frequencies between 42 and 100%. Meristems stored for only 360 days produced shoots when stored in certain culture media. In this sense, the reduction of the strength of



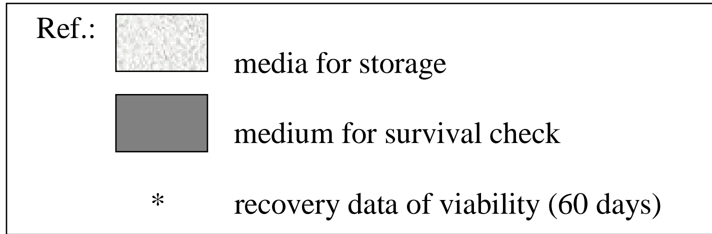


Fig. 1.– Representative scheme of the evaluation procedure used in this work.

the MS to 1/4MS, appears to be one of the more important factors to bear in mind in order to obtain high survival rates of meristems after one year storage. The culture medium composed by 1/4MS + 0.5µM BA (Table 1)

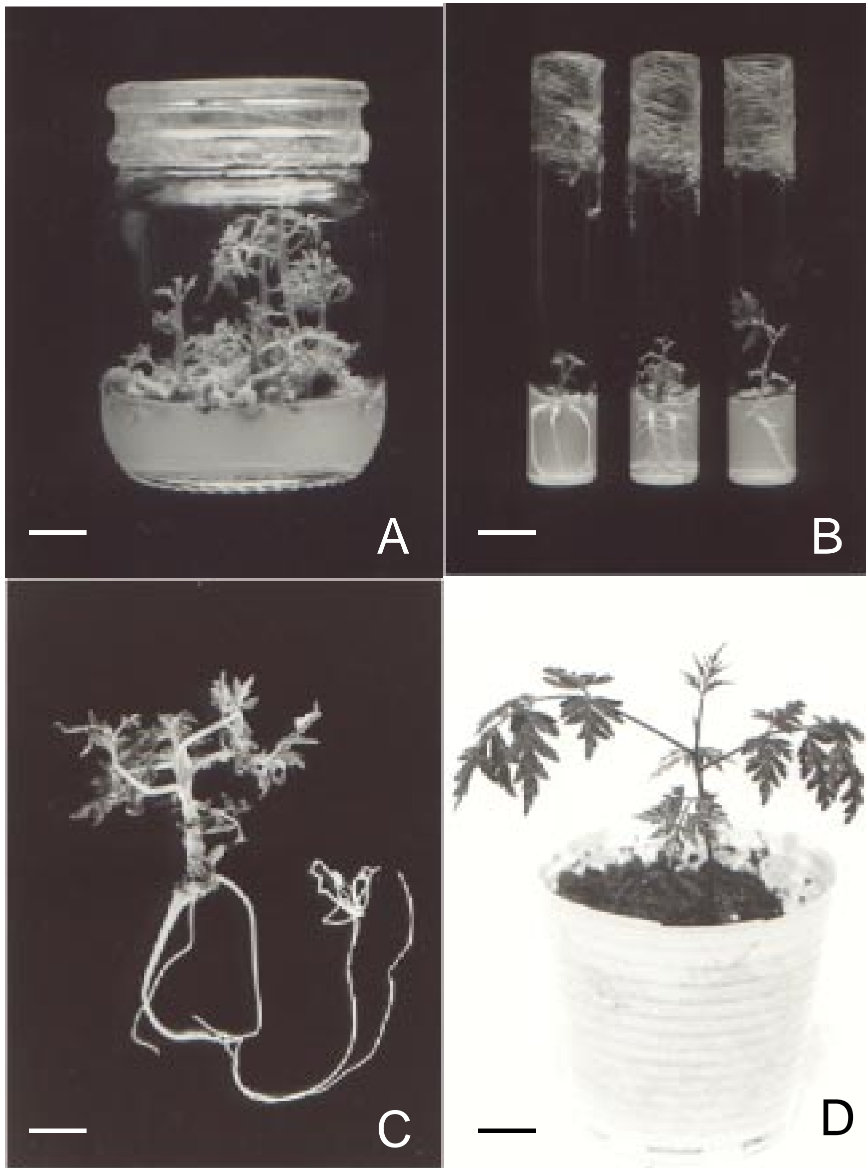


Fig. 2. A.– Induction of multiple shoots from apical meristem-tips of *Melia azedarach* on ¼ MS medium containing 0.5µM BA, recuperated after 12 months at 4ºC (bar = 1cm); B: Rooting of regenerated shoots (bar = 1.5cm); C: plantlet ready for transfer to pot (bar = 0.66 cm); D: One month old acclimatized plant transferred to soil (bar = 1.5 cm).

has allowed that 67% of the explants produce shoots after 60 days in the regeneration medium with 4-5 well developed shoots/explants (Fig 2A). These results are in agreement with those reported for other tropical trees where the reduction of certain components of the culture medium in conjunction with the incubation of the explants at low temperatures have allowed the storage of germoplasm for periods of 1-4 years (5).

Rooting of the regenerated shoots can readily be induced in 60% of the cultures (data not shown) (Fig 2B) and the complete plants obtained (Fig 2C) were successfully transferred to the greenhouse (Fig 2D). However, these results show that both shoot regeneration (67%) and percentage of rooting (60%) are affected by the storage because in the control cultures these values are around 100% (data not shown).

The ANOVA test showed a highly significant difference for the effects of the medium used for conservation (Table 1) and a strong high effect of the duration of storage.

In conclusion, the present investigation shows successful utilization of *in vitro* meristem culture for storage of *Melia azedarach* L. during one year at 4°C without subculture or addition of fresh medium. It is a very simple and effective technique which appears as an alternative to the one reported by (3) which is based in the culture of embryonic axes. The protocol that we recommended consists of:

1) Culture of apical meristem-tips (0.2-0.3 mm in length) on 1/4MS + 0.5μM BA at 4°C.

2) Shoot induction from meristems stored by reculturing them on MS + 2μM BA + 1μM IBA at 27°C.

3) Induction of roots from regenerated shoots by culture on MS + 17.5μM IBA (4 days) and then to MS lacking hormones to differentiate roots.

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